Supplementary information

Netting neutrophils in autoimmune small vessel vasculitis

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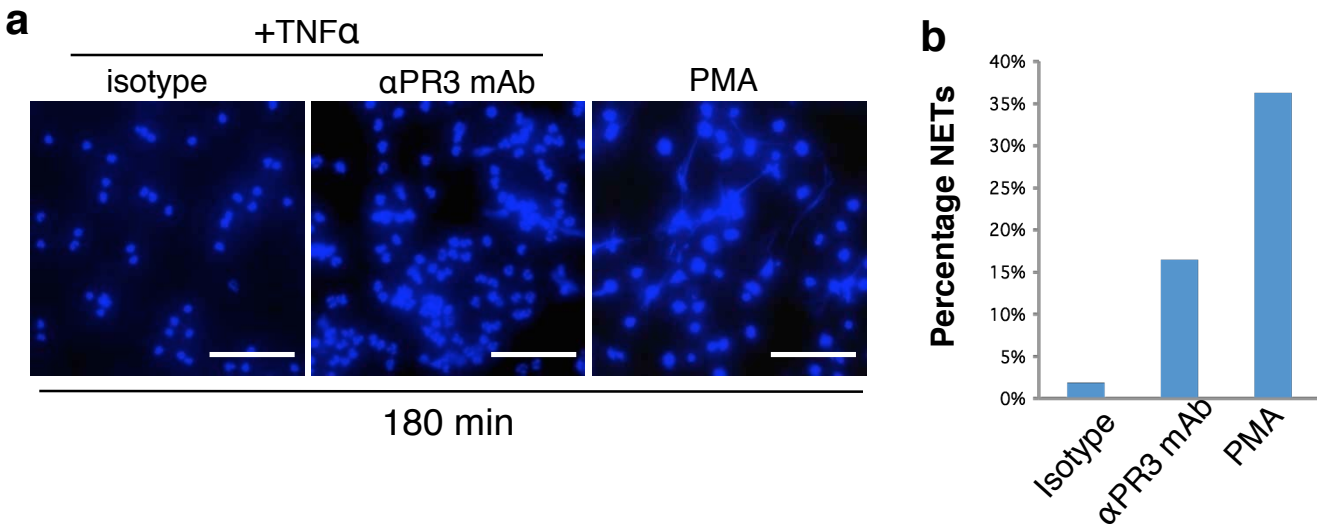
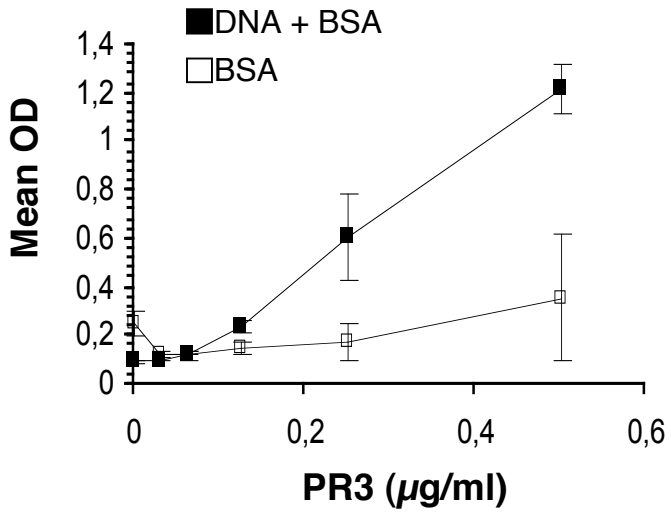
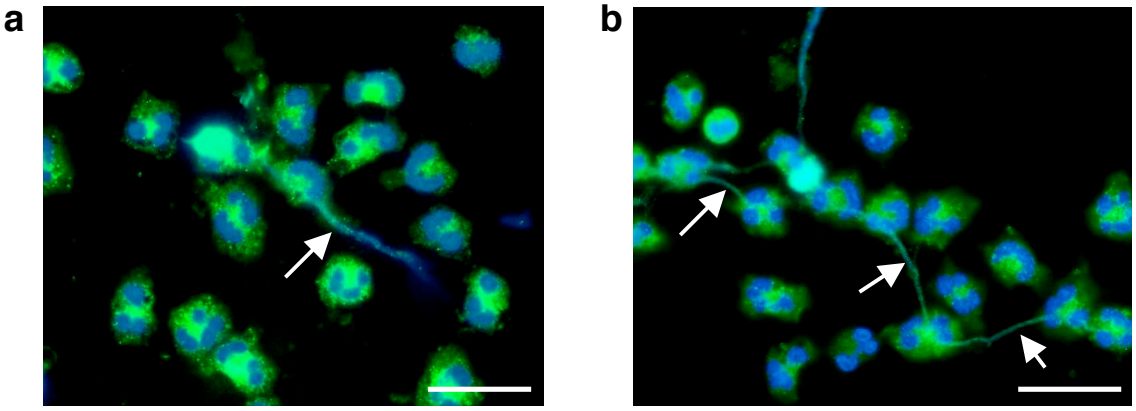


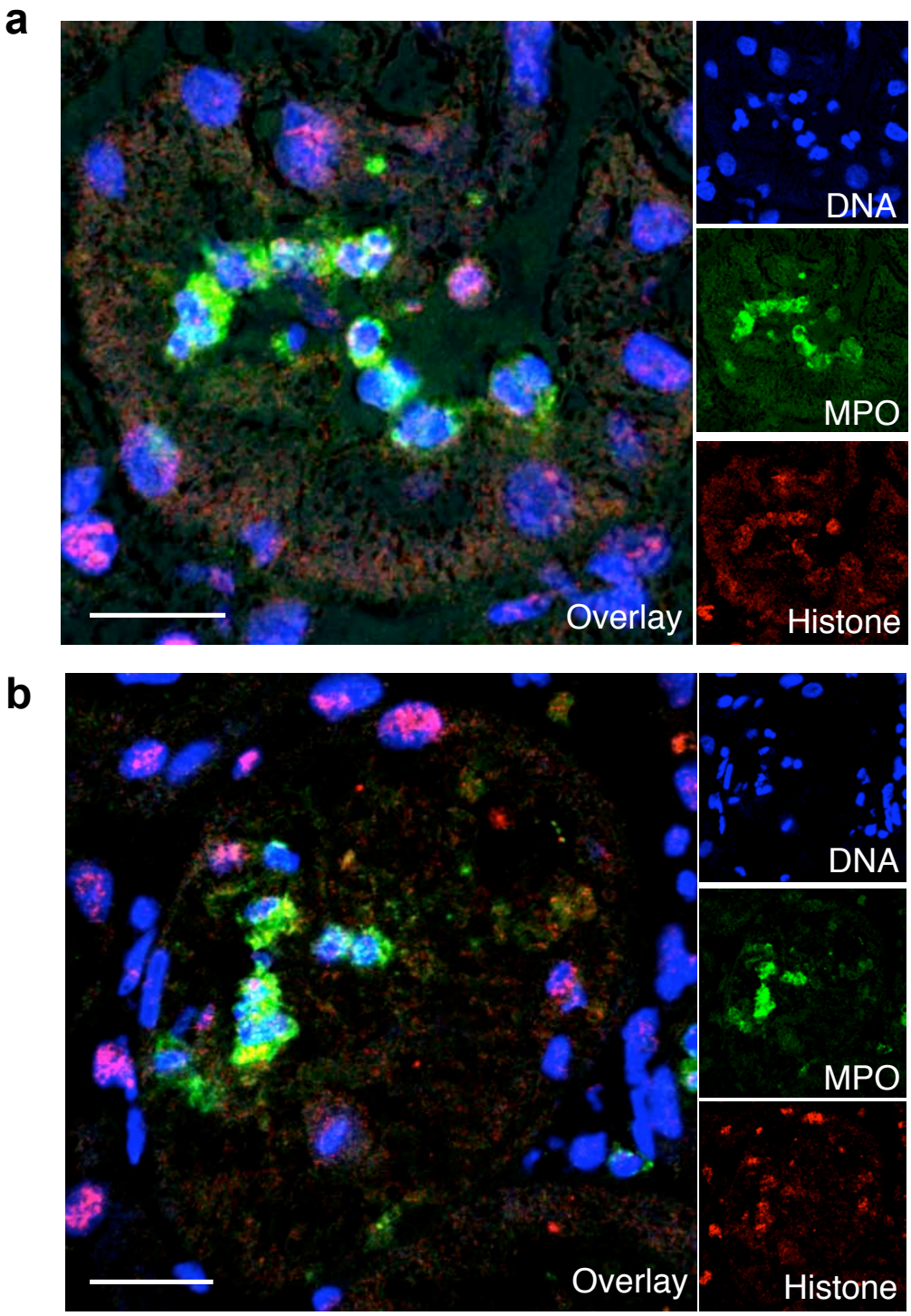
Supplementary Figure 1: NET induction by monoclonal anti-PR3 antibody. Neutrophils were primed with TNFα and incubated with 5 μg/ml mouse monoclonal anti-PR3 antibody (clone 6A6, Wieslab; αPR3 mAb). Mouse isotype matched IgG served as negative control and PMA-activated neutrophils were used as positive control. (**a**) After 180 min of incubation, the DNA release was visualized by fluorescence microscopy of Hoechst 33342-stained specimen; scale bars represent 50 μm. (**b**) Quantitative assessment of the percentage of NET forming cells revealed robust NET formation after αPR3 mAb activation, while incubation with isotype control showed no NET-inducing activity.



Supplementary Figure 2: PR3 binds to DNA. DNA plus bovine serum albumin (BSA) (filled squares) or BSA alone (empty squares) was coated to microtiter plates and incubated with increasing PR3 concentrations to test the DNA binding capacity of PR3. After washing, bound PR3 was identified by a specific immunoassay. PR3 demonstrated significant binding to DNA-containing wells, but not to bovine serum albumin (BSA) coated wells, indicating that PR3 directly binds to DNA.



Supplementary Figure 3: The autoantigen PR3 is accessible to ANCA in sera from SVV patients. Immunofluorescence microscopic analysis of human IgG (green) binding to DNA fibers of NETs (blue) using sera from two WG patients (**a-b**). Both sera show reactivity with PMA-induced NETs to a similar degree, most likely interacting with PR3 on NETs. Scale bar represents 25 μm.



Supplementary Figure 4: *In situ* immunohistochemistry identifying NETs by extracellular MPO (green), histone (red) and DNA (blue) deposits in the capillary tufts of glomerulonephritis biopsies from SVV patients. Scale bar represents 30 μm.

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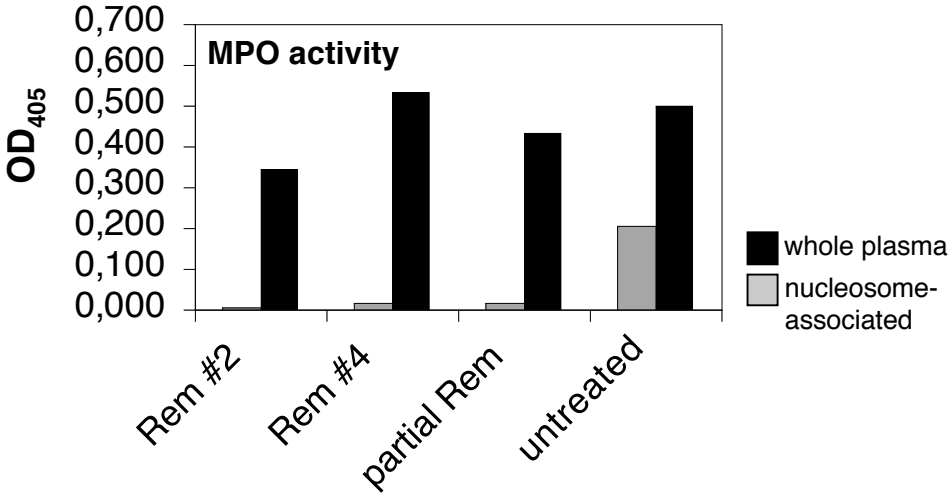
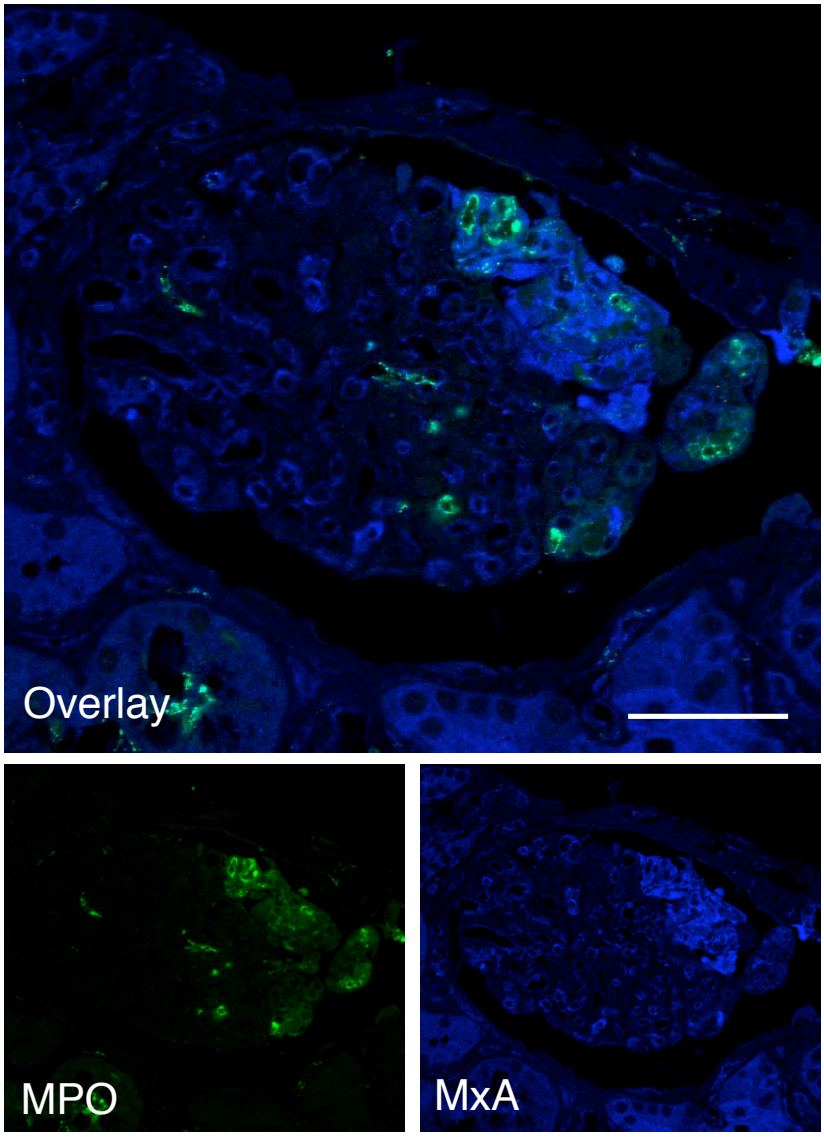


Supplementary Figure 5: To test if circulating nucleosomes were derived from NETs, we used an anti-histone capturing approach to test for nucleosome-associated MPO activity using the TMB substrate. While all samples showed MPO activity in whole plasma, only the specimen from an untreated patient showed MPO activity after anti-histone capture. Patients in remission (Rem) showed no nucleosome-associated MPO activity.



Supplementary Figure 6: MxA expression in close proximity to intraglomerular neutrophil infiltrates. Several MPO (green) positive neutrophils within a necrotic area of the glomerulum are depicted. MxA (blue) is expressed in cells in the immediate vicinity of MPO positivity and in resident glomerular cells. Epithelial cells of distal tubules are strongly positive for Mxa (right lower area). Scale bar equals 25 μm.

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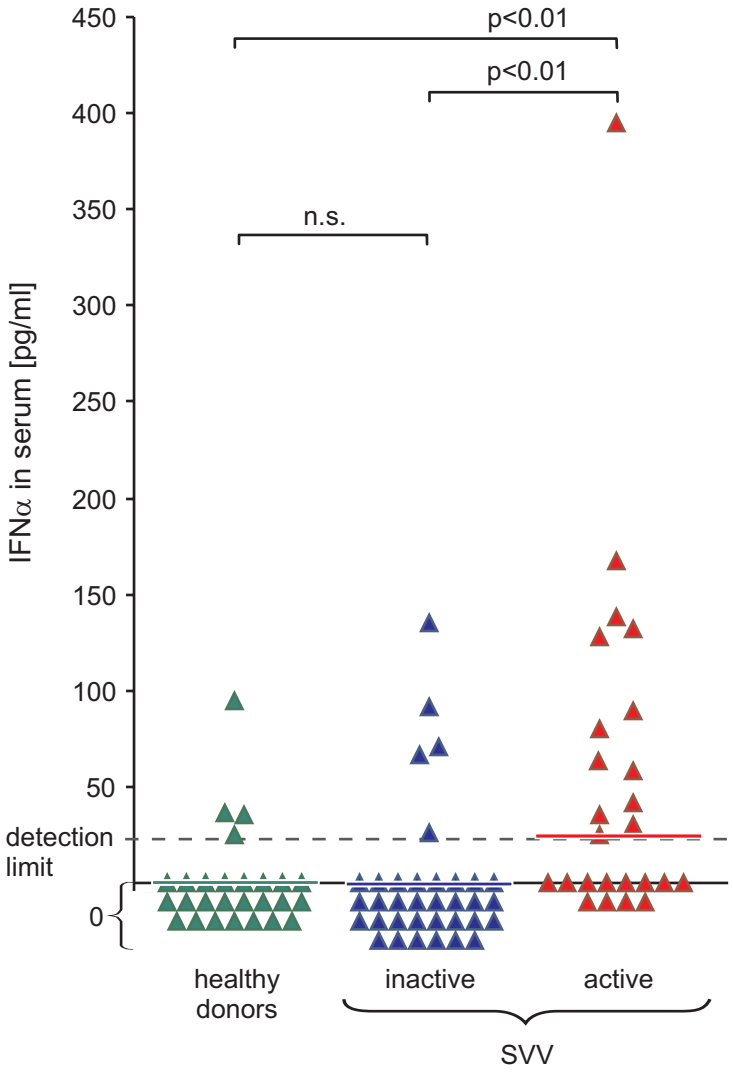

Supplementary Figure 7: Increased levels of IFNα during active SVV. Levels of IFNα were measured by ELISA (Bender MedSystems, Austria) in sera from healthy donors (n = 27) and SVV patients with inactive (n = 35) and with active disease (n = 25). During active SVV, the concentration of IFNα in the circulation was significantly increased (median 24.6 pg/ml, mean 55 pg/ml) when compared to inactive SVV patients (median 0 pg/ml, mean = 11 pg/ml) and healthy donors (median 0 pg/ml, mean = 7 pg/ml). Horizontal bars represent medians. Statistical analysis was done in SPSS using Kruskal-Wallis one way ANOVA on ranks ($p<0.01$) followed by pairwise U-tests (using the Monte Carlo method avoiding influences by ties) with p values corrected for testing of 3 pairs.

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Patient characteristics:

All sera included in this study were from SVV patients that were assessed according to the Birmingham Vasculitis Activity score (BVAS). Complete remission was assumed when the BVAS (Birmingham Vasculitis Activity score) was 0 for at least six months. Active disease was assumed when the BVAS was 1 or greater. Healthy controls were ANCA negative and had no infectious diseases.

Supplementary table 1 Sera included for NET-induction experiments by purified IgG fractions

Patient ID	Age/Gender	Clinical diagnosis	disease state	BVAS	ANCA titer (U/ml)	CRP (mg/dl)	% NETs induction
#1	76/m	WG	rem	0	PR3: <5.0	0,2	**14%**
#5	72/f	MPA	active	8	MPO: 10.3	1,6	**26%**
#9	62/m	WG	rem	0	PR3: <5.0	0,2	**16%**
#10	66/f	WG	rem	0	PR3: 3054	0,3	**26%**
#11	54/f	WG	active	3	PR3: 750	2,7	**34%**
#12	64/m	WG	rem	0	PR3: 166	0,3	**19%**
#17	70/f	WG	rem	0	PR3: 222	0,6	**21%**
#20	75/m	WG	rem	0	PR3: <5.0	0,5	**16%**
#23	66/f	WG	rem	0	PR3: 3395	5,0	**33%**
#29	53/f	MPA	rem	0	MPO: 172	0,1	**20%**
#38	62/f	WG	active	20	PR3: 2070	10,6	**32%**
#49	66/f	WG	rem	0	PR3: 2116	0,2	**26%**

Supplementary table 2 Clinical characteristic of subjects whose kidney biopsies were analyzed for NET deposition.

Patient ID	Age/Gender	Clinical diagnosis	Creatine	IIF	ELISA	Proteinuria	NETs deposition	Neutrophil infiltrates	MxA expression
2203	72/m	WG	>5.5mg%	c-ANCA	PR3+	250 mg/d	**+**	**+**	**+**
2392	68/m	MPA	309 µmol/l	n.a.	MPO+	n.a.	**-**	**+**	**-**
2465	79/m	MPA	3.0 mg%	n.a.	MPO+	1300 mg/d	**+**	**+**	**+**
2543	78/m	WG	1.62mg%	c-ANCA	PR3+	250 mg/d	**-**	**+**	**+**
2842	56/m	WG	1.8mg%	c-ANCA	n.a.	670 mg/d	**+**	**+**	**-**
3149	73/f	MPA	3.2mg%	p-ANCA	n.a.	180 mg/d	**+**	**+**	**+**
3324	77/m	MPA	6.3mg%	n.a.	MPO+	n.a.	**+**	**+**	**+**
3407	66/m	WG	n.a.	c-ANCA	PR3+	700 mg/d	**-**	**+**	**-**
3590	64/m	WG	5.4mg%	ANCA	n.a.	800 mg/d	**+**	**+**	**+**
3648	66/f	MPA	210 µmol/l	p-ANCA	MPO+	900 mg/d	**-**	**-**	**-**
16409/05	65/m	WG	10,4 mg%	n.a.	n.a.	n.a.	**-**	**-**	**n.d.**
18229/05	68/f	WG	11,2 mg%	ANCA	n.a.	1,000 mg/d	**+**	**+**	**n.d.**
30549/05	82/f	MPA	10,2 mg%	pANCA	n.a.	2,100 mg/d	**+**	**+**	**n.d.**
14554/06	66/m	WG	2,9 mg%	cANCA	n.a.	acute renal failure	**-**	**+**	**n.d.**
1654/03	64/f	WG	3,1 mg%	ANCA+	n.a.	2,500 mg/d	**+**	**+**	**n.d.**
							9 / 15	**13 / 15**	**6 / 10**

Supplementary Methods:

Human neutrophils

We isolated human peripheral blood neutrophils by density centrifugation using a Pancoll^{TM} gradient. Briefly, 10 ml blood containing EDTA was then diluted in 10 ml PBS and layered on 10 ml pancoll^{TM}. After 30 min centrifugation at 500 g, we separated neutrophils from the erythrocyte rich pellet by dextran sedimentation. We then eliminated residual erythrocytes by hypotonic lysis and after washing in PBS, resuspended neutrophils in RPMI containing 0.5% low endotoxin bovine serum albumin. Neutrophil purity was routinely ~95% as assessed by forward and side scatter flow cytometric analysis.

ANCA-mediated NET formation

To test if ANCAs trigger the formation of NETs, we activated neutrophils by ANCAs as previously described with some modifications[4]. In brief, we primed neutrophils with 5 ng ml^{-1} TNFα, seeded them on lysinated glass slides and treated them with 250 µg/ml purified IgG from SVV patients (n = 12) and from healthy control individuals (n = 8). Phorbol 12-myristate 13-acetate (PMA) at a concentration of 25 nM served as a positive control. After 180 min, we fixed the cells with 4% PFA and stained the DNA using Hoechst 33342. Using fluorescence microscopy, we determined the percentage of neutrophils releasing DNA fibers from blinded samples in at least five random microscopic fields using a 20× objective.

NETs immunofluorescence

We triggered NET release by isolated neutrophils seeded on lysinated glass slides using 25 nM PMA as described before[9]. After two hours, the cells were fixed using 4% PFA. Unspecific binding sites were blocked in PBS containing 5% goat serum and 1% BSA. To test whether the targeted autoantigens are components of NETs, we incubated the specimens with PR3-specific mouse monoclonal antibody (clone 4A5; Wieslab) and MPO-specific mouse monoclonal antibody (clone 8F4; Abcam). LL37 on NETs was identified using specific mouse monoclonal (clone 3D11; HBT biotechnology). Chromatin was stained using an anti-histone antibody (kindly provided by Prof. M. Monestier). Positive antibody binding was visualized using

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fluorescently-labeled rat anti-mouse IgG secondary antibodies (Pharmingen). DNA was stained using Hoechst 33342.

Identification of NETs and MxA expression *in situ*

Kidney needle biopsies from SVV patients with glomerulonephritis (n = 15) were fixed and embedded in paraffin. 5 µm sections were prepared and mounted on glass slides. After antigen retrieval with citrate buffer, we treated the specimens with blocking buffer and subsequently with primary antibodies against MPO (clone 8F4; Abcam), PR3 (clone 4A5; Wieslab), NE as described previously[7], LL37 (clone 3D11; HBT biotechnology) and histone (kindly provided by Prof. M. Monestier) followed by species-specific secondary antibodies. To detect MxA expression as an indicator of local IFN-α production, we used the affinity purified mouse monoclonal IgG2a antibody directed against human MxA (kindly provided by Dr. G. Koch from the Institute of Virology, University of Freiburg, Germany) as previously described[14]. To identify MxA expression in proximity to neutrophil infiltrates, we co-stained for MPO as mentioned above. Specimens were analyzed on a Leica SP5 confocal microscope and confocal stacks were modeled using Volocity software. Wide field images were recorded using a Nikon DXM 1200 camera on a Leica DMR microscope equipped with band pass filters. Patients specimens were considered NET-positive, when at least 2 areas showed extracellular colocalization of DNA/histone and neutrophil granule markers such as PR3, NE or MPO.

Measuring nucleosome-associated MPO activity

To test for nucleosomes in the serum, we used the commercially available cell death detection ELISA^{PLUS} (Roche, Cat. No: 11774425001). This test is based on the "sandwhich ELISA" principle using two different mouse monoclonal antibodies directed against histones and DNA, respectively. The test was carried out according to the manufacturer´s instructions. Nucleosome-associated MPO activity was measured using a capture ELISA approach: 5 µg ml^{-1} anti-histone monoclonal antibody (Chemicon International) was coated to microtiter plates (75 µl per well) overnight at 4°C. After blocking with 1% BSA (125 µl per well), wells were washed with PBS, and incubated with 40 µl per well of patient and control sera, supernatants from PMA-activated neutrophils as the positive control for NETs, and supernatant from non-activated neutrophils as the negative control. Samples were diluted in incubation

buffer (from commercial kit: Roche Cat. No: 11774425001) to 100 µl final volume per well, incubated for 2 hours at RT while shaking (320 rpm) and washed three times in 200 µl PBS per well. To identify MPO, we added 3.3', 5.5'-tetramethylbenzidine (TMB) as widely used chromogenic substrate for MPO that can be measured via absorbance at 620 nM wavelength, and, after stopping the reaction by the addition of 20 µl 1 M HCl per well, at 450 nm.

Measuring MPO-DNA complexes

To show that circulating nucleosomes in SVV patients are derived from NETosis, we sought to identify granular components of neutrophils in association with circulating nucleosomes. As MPO was found to be a prominent constituent of NETs, we chose to test for MPO attached to nucleosomes. MPO-DNA complexes were identified using a capture ELISA. As the capturing antibody, 5 µg ml^{-1} anti-MPO monoclonal antibody (ABD Serotec, Cat-No. 0400-0002) was coated to 96-well microtiter plates (75 µl per well) overnight at 4°C. After blocking in 1% BSA (125 µl per well), 40 µl of patient sera was added per well in combination with the peroxidase-labeled anti-DNA monoclonal antibody (component No.2 of the commercial cell death detection ELISA kit; Roche, Cat. No: 11774425001) according to the manufacturer´s instructions. After two hours of incubation at RT on a shaking device (320 rpm), the samples were washed three times with 200 µl PBS per well and the peroxidase substrate (ABTS) of the kit (Roche, Cat. No: 11774425001) was added. The absorbance at 405 nm wavelength was measured using Fluostar Optima (BMG Labtech) after 40 min incubation at 37°C in the dark.

DNA binding ELISA

Human DNA was purified from peripheral blood cells of a healthy donor using human DNA extraction Kit (Quiagen) according to the manufactur´s instructions. DNA was coated to 96 well microtiter plates (NUNC) at 5 µg ml^{-1} over night at 4°C. The plate was washed with PBS and blocked with BSA for 4 hrs at 4°C. After another washing step, the coated surfaces were incubated with a dilution series of purified PR3 ranging from 0.5 to 0.0625 µg ml^{-1} in PBS for 2 hrs at RT. Subsequently, PR3 binding after stringent washing with PBS was detected using an immunoassay. Briefly, the surfaces were probed with mouse mAb anti-human PR3 (clone 4A5; Wieslab) following incubation with anti-mouse IgG peroxidase labeled secondary rabbit antibody

(Sigma). Binding was quantified by incubation of the samples with the TMB substrate and colorimetric measurement (FluoOptima; BMG Labtech). DNA binding was determined by direct comparison of DNA-BSA to only BSA coated wells in triplicates.

Interferon-α (IFN-α) ELISA

IFN-α levels were measured in sera from healthy individuals (n = 27), from subjects with inactive SVV (n = 35) and with active SVV (n = 25) using IFN-α capture ELISA (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. All sera included in this study were from SVV patients that were assessed according to the Birmingham Vasculitis Activity score (BVAS).

Statistics

Statistical analysis was done in SPSS using Kruskal-Wallis one way ANOVA on ranks (P<0.01) followed by pairwise U-tests (using the Monte Carlo method avoiding influences by ties) with p values corrected for testing of 3 pairs. A P-value below 0.05 was regarded statistically significant.

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